STIC-ILL MIC RC261.A1 C2

From: Davis, Natalie
Sent: Tuesday, September 10, 2002 3:43 PM
To: STIC-ILL
Subject: 09/901339

Please send:

1. McLesky, et al., 1994, cancer research, 54:523-530

Natalie A.Davis, PhD
Patent Examiner
Art Unit 1642
CM1, Rm 8B13
Mailbox 8E12
Ph (703) 308-6410

[CANCER RESEARCH 54, 523–530, January 15, 1994]

MDA-MB-134 Breast Carcinoma Cells Overexpress Fibroblast Growth Factor (FGF) Receptors and Are Growth-inhibited by FGF Ligands[1]

Sandra W. McLeskey, Ivan Y. F. Ding, Marc E. Lippman, and Francis G. Kern[2]

Lombardi Cancer Research Center [S. W. M., I. Y. F. D., M. E. L., F. G. K.], Department of Biochemistry and Molecular Biology [S. W. M., F. G. K.], De[illegible] of Medicine [M. E. L.], Department of Pharmacology [M. E. L.], Georgetown University Medical Center, Washington, District of Columbia 20007

ABSTRACT

Overexpression of some transmembrane tyrosine kinase growth factor receptors in breast and other tumors has been found to correlate with poor prognosis. Following the cloning of the first two members of the fibroblast growth factor family of receptors (FGFRs), amplification of these receptors in breast carcinomas was found. We have examined 23 breast carcinoma cell lines to determine the extent of expression of mRNA for *fgfrs* 1 through 4. All breast carcinoma cell lines examined expressed mRNA for at least one *fgfr* and several expressed high mRNA levels for a particular receptor. MDA-MB-134, an estrogen receptor-positive cell line, expressed very high levels of mRNA for *fgfr-1* and elevated levels of mRNA for *fgfr-4*. This cell line was found to have an amplified *fgfr-1* gene, but the gene for *fgfr-4* was not amplified. MDA-MB-453 cells were found to express high levels of mRNA for *fgfr-4* without amplification of the gene. MDA-MB-134 cells were examined for their response to FGF ligands. Tyrosine phosphorylation of a M_r 150,000 protein resulted when MDA-MB-134 cells were treated with FGF-1 or FGF-2, implying the presence of a functional FGFR-1. MDA-MB-134 cells were growth-inhibited by picomolar concentrations of FGF-1 or FGF-2 in a dose-dependent manner under both anchorage-independent and anchorage-dependent conditions. These results may provide insight into the consequences of FGFR overexpression in breast tumors and the development of treatment modalities which use manipulation of growth factor responses.

INTRODUCTION

Overexpression of growth factor receptors is thought to be important in the malignant growth of many neoplasms, including breast neoplasms. For example, overexpression of the EGFR[3] or the erbB-2 receptor in breast tumors has been associated with poor prognosis (1–3). Once the FGFRs were identified, gene amplification (4) or overexpression (5) of these receptors in some breast tumors was also detected. However, the significance of this overexpression in the progression of these tumors was not clear. In order to study FGFRs and their relationship to malignant growth, we have examined breast carcinoma cell lines for FGFR overexpression and assessed the effects of FGF ligand stimulation in one cell line which overexpresses mRNA for *fgfrs*.

Four similar genes have been identified which code for FGFRs (6–8). These genes encode structurally similar proteins with an extracellular domain composed of three immunoglobulin loops and an acidic box, a hydrophobic transmembrane domain, and an intracellular domain with a split tyrosine kinase. However, two of these genes, *fgfr-1* and *fgfr-2*, have been shown to have multiple transcripts which arise via alternative splicing (6–10). Splice variants produced from these genes differ with respect to the number of immunoglobulin-like domains in the extracellular region of the receptor and in the sequence for the second half of the third immunoglobulin domain which may arise from alternative exons. In addition, there may be transmembrane and juxtamembrane truncations or deletions which may produce secreted or kinase-deficient protein products (8, 9).

The affinity of the protein products of many of these transcripts for specific FGF ligands has been characterized in transfected cell lines overexpressing one particular receptor isoform and seems to largely depend upon the coding sequence of the third immunoglobulin loop which may be produced by alternative exons (6–8, 11). Recent evidence suggests that the first (12) and second (13) immunoglobulin loops may also participate in the determination of binding affinity for particular FGF ligands. Several *fgfr-1* transcripts produce proteins which have similar affinity for FGF-1 (acidic FGF) and FGF-2 (basic FGF) as well as FGF-4 (K-FGF or HST-1). However, the product of one transcript of the *fgfr-1* gene has a higher affinity for FGF-1 than for other FGFs tested (8). Another transcript of the *fgfr-1* gene encodes a truncated receptor lacking transmembrane or intracellular domains. This protein, which is secreted, exhibits higher affinity for FGF-2 (14). The protein products of some transcripts of *fgfr-2* have similar affinity for FGF-1 and FGF-2, but exon shuffling within the third immunoglobulin loop produces a different transcript whose product exhibits preferential affinity for FGF-7 (KGF) and FGF-1 with much lower affinity for FGF-2 (6–8, 15). Alternative transcripts of *fgfr-3* have not been characterized, and the protein product has high affinity for FGF-1 (6–8, 16). The only known protein product of *fgfr-4* has much higher affinity for FGF-1 than FGF-2 (6–8). Recently, the cDNA for a new type of cysteine-rich FGFR that does not contain a tyrosine kinase domain has been identified, the product of which binds FGF-1, FGF-2, and FGF-4 with high affinity (17). Thus, at present, all known membrane-bound FGFRs bind FGF-1 with high affinity, although many have reduced affinity for FGF-2 and most have reduced affinity for FGF-7 (6–8, 17).

As mentioned, affinities of FGFRs for FGF ligands have usually been characterized using transfected cells expressing one particular *fgfr* cDNA. Given the multiplicity of FGFR genes and transcripts and the lack of specificity of many of their protein products for particular FGFs, it is difficult to assess the action of a specific ligand on a specific receptor in systems where more than one receptor and/or ligand may be present. However, even though *in vivo* different FGF ligands may be expressed in a tissue-specific manner and stimulate specific FGFRs present in that tissue, using an *in vitro* cell culture system, we can activate all known membrane-bound FGFRs using FGF-1 as a ligand. Therefore, at least some of the consequences of FGFR overexpression can be investigated *in vitro* by identifying cell lines which overexpress specific FGFRs and stimulating them with FGF-1. It must be kept in mind, however, that the consequences of ligand stimulation of a particular receptor also may depend upon other features of that receptor besides ligand affinity. These factors may include the following: the tyrosine kinase activity of the FGFR; its ability to form homo- or heterodimers with other FGFRs, which may also include splice variants capable of acting in a dominant negative

Received 7/19/93; accepted 11/15/93.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by NIH grants CA50376 (to F. G. K.) and CA58185 (to M. E. L.). S. W. M. is supported by NCI postdoctoral fellowship CA09218.

[2] To whom requests for reprints should be addressed, at Lombardi Cancer Research Center, Room S107, Georgetown University Medical Center, 3800 Reservoir Rd., N.W., Washington, DC 20007.

[3] The abbreviations used are: EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FGF, fibroblast growth factor; EGF, epidermal growth factor; BSA, bovine serum albumin; FBS, fetal bovine serum; IMEM, improved minimal essential medium; SDS, sodium dodecyl sulfate; SSC, standard saline citrate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; cDNA, complementary DNA; PCR, polymerase chain reaction; TBS-T, 10 mM Tris, pH 7.5–150 mM NaCl-0.02% Tween 20; EC_{50}, median effective concentration.

fashion; its ability to bind to particular intracellular effectors; and the presence or absence of other downstream effector molecules. These properties of the different FGFR protein products are just beginning to be characterized (11). In addition, the level of tyrosine phosphatase activity in dephosphorylating tyrosine residues involved in receptor-substrate binding or on phosphorylated substrates is also likely to be involved in modulating the response to ligand stimulation.

We have examined breast carcinoma cell lines for transcripts of *fgfr*s 1 through 4 by RNase protection analysis in order to characterize the range of expression of particular receptors and to examine the FGF-stimulated growth characteristics of overexpressing cell lines. We have characterized one breast carcinoma cell line, MDA-MB-134, which overexpresses mRNA for *fgfr-1* and also has high levels of *fgfr-4* mRNA expression. This cell line is growth-inhibited by FGF ligands, reminiscent of the EGFR-overexpressing breast carcinoma cell line MDA-MB-468, which is growth-inhibited by EGF (18). Our results may provide insight into the mechanism of FGFR signal transduction or the mechanism by which overstimulation of a growth factor receptor signal transduction pathway may produce growth inhibition.

MATERIALS AND METHODS

Materials. Recombinant human FGF-2 (Upstate Biotechnology, Inc., Lake Placid, NY) was purchased in a sterile solution. Lyophilized recombinant human FGF-1 (Upstate Biotechnology, Inc.) was dissolved in a sterile solution containing 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Sigma Chemical Co., St. Louis, MO) and 0.5% BSA (Sigma). The 355-base pair cDNA fragment of FGFR-1 used in RNase protection assays and Southern blot analyses was kindly provided by Dan Johnson, University of California, San Francisco, CA (19). Vectors containing sequences of FGFR-3 and FGFR-4 used for construction of riboprobes were kindly provided by Kari Alitalo, University of Helsinki, Helsinski, Finland (20).

Cell Lines. All cell lines used except MCF-7 were obtained from American Type Culture Collection and grown in improved IMEM (Biofluids, Rockville, MD) with 5% or 10% FBS at 37°C in a humidified, 5% CO_2 atmosphere. MCF-7 cells were obtained from the laboratory of Marc Lippman and grown in IMEM-supplemented with 10% FBS.

Southern Analysis. High molecular weight DNA was prepared by incubating cell lysates overnight at 37°C in a solution of 0.01 M Tris-Cl, pH 7.9, 0.01 M EDTA, 100 μg/ml proteinase K, and 0.6% SDS. After adjusting the sodium chloride concentration to 0.15 M, the lysates were successively extracted with equal volumes of Tris-equilibrated phenol, pH 8.0; phenol-chloroform-isoamyl alcohol (25:24:1); and chloroform-isoamyl alcohol (24:1). DNA was precipitated with 0.1 volume of 3 M sodium acetate, pH 5.0, and 2.5 volumes 100% ethanol, washed with 70% ethanol, dried, and resuspended in 1 mM Tris-Cl, pH 7.4–0.5 mM EDTA. DNA was digested with appropriate restriction enzymes as indicated and subjected to electrophoresis in a 1% agarose gel. Ethidium bromide staining was used to demonstrate equal loading and complete digestion. DNA was partially depurinated with 25 mM HCl and transferred to a nylon membrane (Zetabind; Cuno, Inc., Meriden, Connecticut) using a 0.4 M NaOH solution. After baking at 80° for 1 h, blots were washed in 15 mM sodium chloride-1.5 mM sodium citrate, pH 7.0 (0.1× SSC = 150 mM sodium chloride-15 mM sodium citrate), 0.5% SDS at 65°C, prehybridized for 3 h at 65°C in 750 mM NaCl, 5 mM EDTA, 50 mM NaH_2PO_4, pH 7.4 (5× saline-sodium-phosphate-EDTA); 0.05% Ficoll, 0.05% polyvinylpyrrolidone, 0.05% BSA (1X Denhardt's solution); 0.2% SDS, 5% dextran sulfate, and 500 μg/ml sonicated salmon sperm DNA, and hybridized overnight at 65° in 5X saline-sodium-phosphate-EDTA, 1X Denhardt's solution, 0.2% SDS, 10% dextran sulfate, 100 μg/ml salmon sperm DNA, plus 1–2 million dpm random-primed, ^{32}P-labeled probe/ml. The blots were washed twice at room temperature with 5 mM sodium phosphate solution, pH 7.0, 1 mM EDTA, and 0.2% SDS, and twice at 65°C with 0.1X SSC and 0.1% SDS, followed by autoradiography.

Synthesis of Probes. A Bluescript KS plasmid containing a *Hin*cII-*Ava*I, 355-base pair fragment of the *fgfr-1* cDNA (nucleotides 1068 to 1424; accession no. M34641) in the transmembrane region, was used to prepare both single- and double-stranded probes. A 229-base pair cDNA sequence of *fgfr-2* containing nucleotides 1851 through 2080 (kinase insert domain; accession no. X52832) was obtained using an antisense oligonucleotide primer for nucleotides 2061 through 2080 with an 8 base *Bam*HI restriction site appended to its 5′ end to produce a single-stranded cDNA fragment by reverse transcription from 2 μg total RNA extracted from MCF-7 cell lysates using a reverse transcriptase-PCR kit (GeneAmp RNA PCR Kit; Perkin Elmer Cetus, Norwalk, CT). PCR (1 min at 95°C and 1 min at 60°C for 35 cycles, followed by 7 min at 60°C) was used to amplify this fragment using the same antisense primer and a sense primer to nucleotides 1851 through 1869 with an *Eco*RI restriction site appended to its 5′ end. The PCR-amplified fragment was purified, digested with *Bam*HI and *Eco*RI, and ligated into similarly digested pGEM7Zf(+) (Promega, Madison, WI). The sequence of the *fgfr-2* cDNA fragment thus obtained was verified by dideoxy sequencing (21).

Plasmids containing cDNA sequences for *fgfr-3* (JTK4) and *fgfr-4* (JTK2) in pGEM3 (Promega) were kindly provided by Kari Alitalo. The *fgfr-3* plasmid was digested with *Sma*I, and a fragment containing the plasmid sequences and nucleotides 1369 through 1757 (accession no. M58051; tyrosine kinase domain) of the *fgfr-3* cDNA was purified and recircularized. In a similar manner, the *fgfr-4*-containing plasmid was digested with *Pst*I, and a fragment consisting of the plasmid sequences plus *fgfr-4* cDNA nucleotides 199 through 387 (accession no. X57205; a region encompassing the first immunoglobulin loop in the extracellular domain) was purified and recircularized. The plasmid used for the probe for GAPDH has been described previously (22).

Antisense ^{32}P-labeled riboprobes for *fgfr-1*, *fgfr-2*, *fgfr-3*, *fgfr-4*, and GAPDH were prepared as described (23). Random primed probes for *fgfr-1* or *fgfr-4* used in Southern blotting were prepared from a 355-base pair cDNA fragment of *fgfr-1* purified from the plasmid described above or a 2.7-kilobase pair cDNA fragment of *fgfr-4* purified from the plasmid JTK2. Fifty μCi of each of two labeled nucleotides ([α-^{32}P]dATP and [α-^{32}P]dCTP; 3000 Ci/mmole; Amersham, Arlington Heights, IL) were evaporated to a volume of 7 μl. Reaction buffer was added along with 50 nM unlabeled dGTP and TTP and 20 ng denatured template DNA. Two units Klenow fragment (all reagents from Boehringer-Mannheim random-primed probe kit, Indianapolis, IN) were added to a total volume of 10 μl. The reaction mixture was incubated for 2 h at 37°C followed by heating at 68°C for 15 min. The volume was adjusted to 100 μl in 100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA [100 mM NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0) buffer] and the probe was separated from unincorporated nucleotides using a G-50 Sephadex spin column (Quick Spin Columns; Boehringer-Mannheim).

RNase Protection. Total RNA from various cell lines was hybridized with a cocktail of ^{32}P-labeled antisense riboprobes for *fgfr-1* (355-base pair protected fragment), *fgfr-2* (229-base pair protected fragment), *fgfr-3* (388-base pair protected fragment), *fgfr-4* (188-base pair protected fragment), and GAPDH (104-base pair protected fragment) as described (23). Pilot experiments performed with individual riboprobes failed to indicate overlapping digestion products that would preclude the use of multiple probes in a single assay. Samples were subjected to polyacrylamide gel electrophoresis (6% polyacrylamide, 7 M urea, Gel-Mix 6; Gibco-BRL, Gaithersburg, MD) followed by autoradiography.

Growth Curves. MDA-MB-134 cells were plated at a density of 50,000 cells/well in 24-well plates (Costar, Cambridge, MA) in IMEM plus 10% FBS. Because of a low plating efficiency, this usually resulted in about 20,000 cells/well at the beginning of the growth assay. MDA-MB-453 cells were plated at a density of 10,000 cells/well in IMEM plus 5% FBS. For growth assays involving estrogen-depleted or serum-free medium, cells were subjected to at least four media changes over a 24-h period the day following plating. Cells in one set of wells were counted on day 0, and medium was changed in all other wells to appropriate treatments. Cells were counted every 2–5 days by detaching the cells with 1 ml 10 mM EDTA in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.1 mM KH_2PO_4, pH 7.4), transferring the contents of each well to a cuvette containing 10 ml counting buffer (Isoton III; Coulter, Hialeah, FL), and counting using a Coulter counter.

Soft Agar Assays. One ml of a mixture of 0.6% agar (Bacto-agar; Difco, Detroit, MI) in IMEM with 5% FBS was plated on the bottom of 35-mm tissue culture dishes (Costar) and allowed to harden. Ten thousand cells per dish were suspended in 0.8 ml of a top agar solution of 0.36% agar in IMEM with 5% FBS and appropriate treatments. Dishes were allowed to harden and then incubated at 37°C in a 5% CO_2 atmosphere for 2–3 weeks. Colonies greater

than 60 μm in diameter were counted using an Omnicon 3600 Image Analysis System (Dynatech Laboratories, Webster, NY).

Western Blot for Phosphotyrosine. Cells were plated at a density of 200,000 cells/well in 24-well plates and allowed to attach overnight. Cells were placed in IMEM without additives for 1 h and then treated with indicated concentrations of FGF-1 plus 50 μg/ml heparin sulfate (average *M*, 8000; Sigma), FGF-2, or no treatment in IMEM for 30 min at room temperature. Treatments were aspirated, and cells were lysed with 100 μl loading buffer (Sepasol; Enprotech, Natick, MA). Protein concentrations were determined using a colorimetric procedure (Bio-Rad, Richmond, CA), and 26 μg protein from each sample was loaded onto a 4–20% SDS-polyacrylamide minigel (Novex, San Diego, CA). Proteins were transferred to nitrocellulose electrophoretically using 300 mA for 1 h. Blots were blocked overnight in a solution of TBS-T (FisherBiotech, Pittsburgh, PA) with 5% BSA. Primary antibody (2 μg/ml; mouse monoclonal antiphosphotyrosine, no. 05–321; UBI, Lake Placid, NY) was applied to the blot in a solution of TBS-T with 0.1% BSA for 3 h at room temperature. The blot was washed for five 10-min washes with TBS-T, and a secondary antibody (goat anti-mouse IgG coupled to alkaline phosphatase; no. S3721; Promega) 1:7500 in TBS-T was applied for 1 h. The blot was washed as before, and color reagent (165 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate and 330 μg/ml nitroblue tetrazolium, both from Promega, in 100 mM Tris, pH 9.5–1 M NaCl-125 mM MgCl) was applied until sufficient color developed.

RESULTS

MDA-MB-134 and MDA-MB 453 Cells Have Elevated Levels of mRNA for FGF Receptors. We have developed plasmids containing templates for antisense riboprobes (Fig. 1) which detect transcripts of four *fgfr* genes. Our probes for *fgfr-1* and *fgfr-2* will detect all transcripts thus far described except the truncated and/or secreted receptor mRNAs (6–8). The probes for *fgfr-3* and *fgfr-4* presumably will detect all transcripts since only one transcript for each has been described. We performed screening RNase protection assays on total RNA from 23 breast carcinoma cell lines to determine the levels of expression of particular *fgfr* transcripts (Fig. 2; Table 1). Three of the cell lines (13%) expressed high levels of mRNA for *fgfr-1* with MDA-MB-134 expressing very high levels. Barely detectable expression of *fgfr-1* mRNA was observed in 9 of the cell lines (39%). Our *fgfr-1* riboprobe will detect one splice variant of *fgfr-1*. Fragments with sizes of 216 base pairs and 133 base pairs are produced from hybridization of the probe with mRNA which lacks nucleotides 1285–1290 and codes for a protein in which a threonine and valine are deleted in the juxtamembrane domain (9). All of the cell lines which express significant *fgfr-1* mRNA also express this *fgfr-1* splice variant mRNA. Moreover, the ratio of signal obtained from the full-length protected probe compared to the signal obtained from the protected fragments produced by the splice variants seems similar in all cell lines in which the splice variant-protected fragment is detectable. The FGF-7 receptor is encoded by mRNA produced from the *fgfr-2* gene which contains coding sequence for the third immunoglobulin loop, which is produced from an alternative exon (10, 15). Our probe spans a region which is common to mRNAs encoding the FGF-7 receptor and other transcripts of *fgfr-2*. Since FGF-7 has been reported to be a mitogen for epithelial cells (24), we were interested in determining whether most breast carcinoma cell lines produced transcripts hybridizing to our *fgfr-2* probe. However, only 1 (4%) of the cell lines, T47D, expressed high levels of *fgfr-2* mRNA, and only 8 of the cell lines (35%) produce *fgfr-2* transcript levels detectable without prolonged exposure of the autoradiogram. No cell line expressed high levels of *fgfr-3* transcripts, and transcripts for *fgfr-3* mRNA above barely detectable levels were present in only 5 (22%) of the cell lines tested. Five of the cells lines (22%) expressed high levels of *fgfr-4* mRNA with the large majority of cell lines (19 of 23, 83%) expressing easily detectable amounts of *fgfr-4* mRNA.

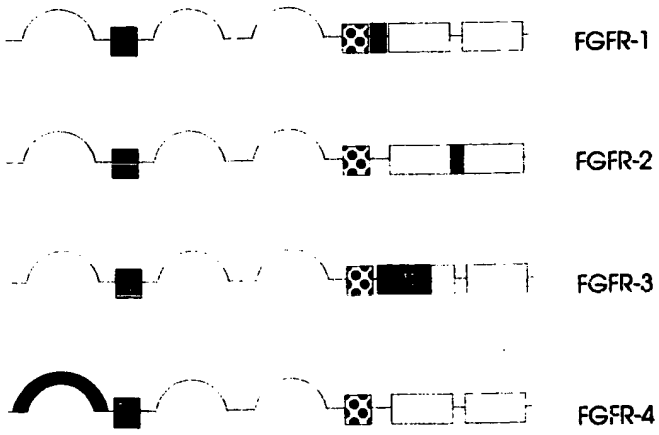

Fig. 1. FGF receptor probes used in RNase protection assays. Probes used in RNase protection were synthesized to detect transcripts of the indicated *fgfrs*. ∩, immunoglobulin loop; ■, acidic box; , transmembrane domain; □, tyrosine kinase domain; □, area covered by probe.

In contrast to FGF ligands, which seem to be mostly expressed in estrogen receptor-negative breast carcinoma cell lines (25), expression of mRNA for FGFRs seems to be relatively ubiquitous among all cell lines, and patterns of overexpression based on a particular phenotype are not apparent. T47D cells express relatively high levels of mRNA for all four FGFRs. FGFR-4 mRNA was expressed at easily detectable levels most consistently. (Specific activity of the FGFR probes was approximately equal.)

Because MDA-MB-134 cells express very high levels of specific *fgfr* transcripts, we chose to examine the genetic and biological characteristics of these cells. MDA-MB-134 is an estrogen receptor-positive breast carcinoma cell line (26) which produces very high levels of *fgfr-1* mRNA and moderate levels of *fgfr-4* mRNA (Fig. 2; Table 1).

MDA-MB-134 Cells Have an Amplified *fgfr-1* Gene but the *fgfr-4* Gene Is Not Amplified. In order to determine whether the increased levels of *fgfr-1* or *fgfr-4* mRNA observed in MDA-MB-134 or MDA-MB-453 cells were the result of increased transcription of one gene or multiple copies of the gene, we analyzed high molecular weight DNA prepared from those two cell lines as well as DNA from cell lines expected to have a normal number of copies of each gene and placenta DNA (Fig. 2; Table 1). Southern blot analysis of *Eco*RI- and *Bam*HI-digested DNA using a 355-base pair cDNA probe containing 15 base pairs from the 3′ end of exon 7, all of exon 8, and all but 9 base pairs of exon 9 (10) revealed that MDA-MB-134 DNA contains approximately 10–15 copies of the *fgfr-1* gene (Fig. 3, *A* and *B*). In addition, the pattern of bands produced from an *Eco*RI digest of DNA from MDA-MB-134 cells is different from what has been reported previously. We have obtained three bands at approximately 12, 10, and 7 kilobase pairs. The 10-kilobase pair band is equal in intensity to the 12-kilobase pair band in MDA-MB-134 cells but not in the other DNA digests. However, the 10-kilobase pair band is present upon prolonged exposure of the autoradiogram in all cell lines tested. Ruta *et al.* (27), using a 2-kilobase pair cDNA probe which spanned the entire coding region of *fgfr-1*, have reported 12-kilobase pair and 6.9-kilobase pair bands in human lymphoblastoid cells. Adnane *et al.* (4), using a 650-base pair *Eco*RI-*Hind*III probe which may have some sequences in common with ours, have reported 10-kilobase pair and 4-kilobase pair bands in human leukocytes and breast tumor specimens. The presence of different-sized bands reported by different authors can possibly be explained by the presence of different alleles in different individuals. However, the difference in intensity of the 10-kilobase pair band when compared with the 12-kilobase pair band between different cell lines indicates that the MDA-MB-134 cells may possibly contain an amplification in which different parts of the *fgfr-1*

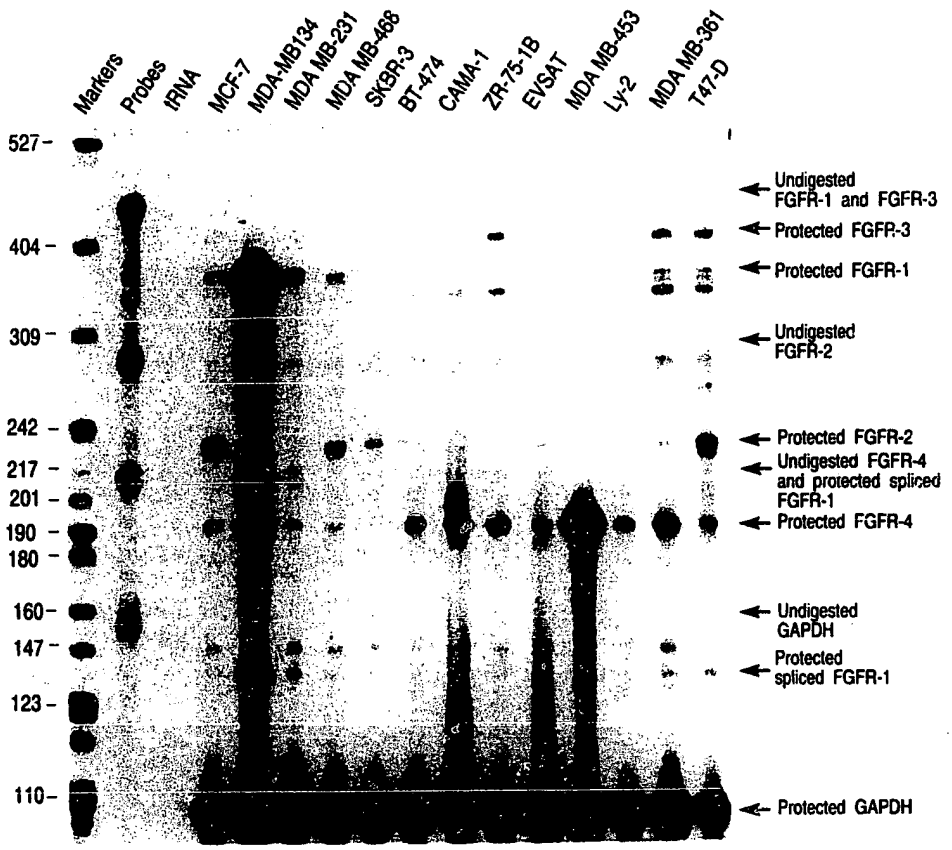

Fig. 2. RNase protection assays of breast carcinoma cell lines for FGFR mRNA. RNase protection assays were performed on 30 μg total RNA from the indicated cell lines or tRNA using a cocktail of all four *fgfr* riboprobes of approximately equal specific activity and a riboprobe for GAPDH. Intact probes and protected fragments are as indicated.

Table 1 *Summary of data from RNase protection assays*

Cell line	FGFR-1	FGFR-2	FGFR-3	FGFR-4
Estrogen-receptor positive				
MCF-7	+[a]	++	+/–	+
LY-2	+	+/–	+	+
T47D	+++	+++	++	++
ZR75–1	+	+/–	+	++
MDA-MB-134	>++++	++	+/–	+++
MDA-MB-175	+	+/–	+/–	+
CAMA-1	+/–	+/–	+/–	+++
BT474	+/–	+/–	+/–	++
Estrogen-receptor negative				
BT20	+/–	++	+/–	+
BT483	+++	+	++	+++
BT549	+	+/–	+/–	+/–
ZR75–30	+/–	+	+/–	++
MCF-7 ADR	+	+/–	+/–	++
MDA-MB-157	++	+/–	+/–	++
MDA-MB-231	++	+/–	+/–	+
MDA-MB-361	+	+/–	+	+++
MDA-MB-435	+/–	+/–	+/–	+/–
MDA-MB-436	+/–	+/–	+/–	+/–
MDA-MB-453	+/–	+/–	+/–	>++++
MDA-MB-468	+	+	+/–	+
SK-Br-3	+/–	+	–	+/–
HS578t	+	+/–	+/–	+
Evsa T	+/–	+/–	+/–	+

[a] –, no detectable band even upon prolonged exposure of the autoradiogram; +/–, barely detectable band upon prolonged exposure; +, detectable band upon overnight exposure; ++, easily detectable band upon overnight exposure; +++, heavy band upon overnight exposure; ++++, very heavy band upon overnight exposure. Autoradiograms were exposed for very short periods of time to reveal the differences in loading as revealed by the intensity of the GAPDH band. Scores were then adjusted for differences in loading. These data were obtained from two RNAse protection experiments (one of which is depicted in Fig. 2) using a cocktail of four FGFR probes. In addition, at least one pilot experiment using a single probe was done for each FGFR. Data obtained from the multiple probe experiments confirmed data obtained from single probe experiments.

gene are amplified differently. The gene for *fgfr-1* has been reported to be on chromosome 8 (27). Interestingly, other authors have found monosomy for chromosome 8 in MDA-MB-134 cells (28). Southern blot analysis of MDA-MB-134 and MDA-MB-453 genomic DNA revealed no amplification of the *fgfr-4* gene in either case (Fig. 3*C*). (We observed the same bands at approximately 4.3 kilobase pairs and 2.3 kilobase pairs in this blot as have been reported (20). However, the higher molecular weight band in the placental DNA seems to migrate slightly slower than corresponding bands in the cell lines. In addition, the 2.3-kilobase pair band is missing from the placental lane. Presumably, this represents alleleic variation.) Thus, high levels of transcripts for *fgfr-1* in MDA-MB-134 cells can be explained at least partially by gene amplification. However, the overexpression of *fgfr-4* mRNA in both MDA-MB-134 and MDA-MB 453 cells must be due to increased transcription or to a prolonged half-life of *fgfr-4* mRNA.

Stimulation of MDA-MB-134 Cells with FGF Ligands Produces Antiphosphotyrosine Immunoreactivity Having a Molecular Size Consistent with FGFR-1. To determine whether FGF receptor proteins in MDA-MB-134 cells were functional, we assessed their ability to produce ligand-stimulated tyrosine phosphorylation. Following treatment of MDA-MB-134 cells with FGF-1 or FGF-2, cell lysates were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with an antiphosphotyrosine antibody. Lysates of FGF-stimulated MDA-MB-134 cells exhibited bands of antiphosphotyrosine immunoreactivity with a molecular size of about 150,000, consistent with the reported size of FGFR-1 (29). This band was absent in untreated lysates (Fig. 4). A204 cells, a rhabdomyosarcoma cell line which has been reported to overexpress FGF receptors (Refs. 29 and 30; Fig. 4); MDA-MB-453 breast carcinoma cells, which express large amounts of *fgfr-4* mRNA; and MCF-7 breast carcinoma cells all failed to exhibit detectable FGF-stimulated antiphosphotyrosine immunoreactivity in this assay (data not shown). Thus, antiphosphotyrosine immunoreactivity detected in lysates of MDA-MB-134 cells following FGF stimulation implies the presence of functional FGF receptors capable of autophosphorylation.

MDA-MB-134 Cells Respond to FGF-1 and FGF-2 in Picomolar Concentrations by Growth Inhibition. To determine whether the FGFR proteins in MDA-MB-134 cells were associated with a biological response, we tested the effect of FGF-1 and FGF-2 on their growth. In the presence of 10% FBS, added FGF-1 or FGF-2 was inhibitory to the growth of MDA-MB-134 cells in a dose-dependent fashion (Fig. 5, *left* and *middle*) in an anchorage-dependent growth assay. When these cells were grown in estrogen-depleted medium (phenol-red-free IMEM supplemented with 5% charcoal-stripped calf serum) or serum-free medium, essentially no growth occurred and added FGF-1 (16 pM to 5.4 nM) or FGF-2 (5.7 pM to 5.7 nM) had no effect (data not shown). In estrogen-depleted medium, growth stimulated by 10 pM 17β-estradiol was also attenuated by 162 pM FGF-1 treatment (data not shown). The dose-dependent inhibitory effects of FGF-1 were additive to those produced by 3 nM 4-OH-tamoxifen (a maximally inhibiting dose of 4-OH-tamoxifen for these cells under these conditions) in medium containing 10% FBS (Fig. 5, *right*). It should be noted that the EC_{50} for the growth inhibition produced by either FGF-1 or FGF-2 is in the range of 150–200 pM, which is only slightly higher than the published K_d for binding of FGF-1 or FGF-2 (7) to FGFR-1 and is an order of magnitude greater than the EC_{50} published for growth stimulatory effects of FGF-1 or FGF-2 (31) in other cells. In an anchorage-independent growth assay in medium containing 10% FBS, a similar dose-dependent growth inhibition was observed upon treatment with either FGF-1 or FGF-2 (Fig. 6). Thus, FGF-induced growth-stimulation was never observed in MDA-MB-134 cells under any conditions tested and growth-inhibition was observed when the cells were actively growing, whether that growth was stimulated by 17-β estradiol or FBS. The dose-response curve for growth inhibition produced by either ligand is not biphasic (Fig. 5, *insets*) as has been observed for the growth-inhibitory effects of EGF on the EGFR-overexpressing cell line, MDA-MB-468. In addition, it has been reported that EGF-treated MDA-MB 468 cells can be "rescued" from the growth inhibitory effects of EGF by inclusion of high levels of glucose in their growth medium (18). This manipulation has no effect on the growth-inhibitory effects of FGF-1 or FGF-2 on MDA-MB-134 cells (data not shown).

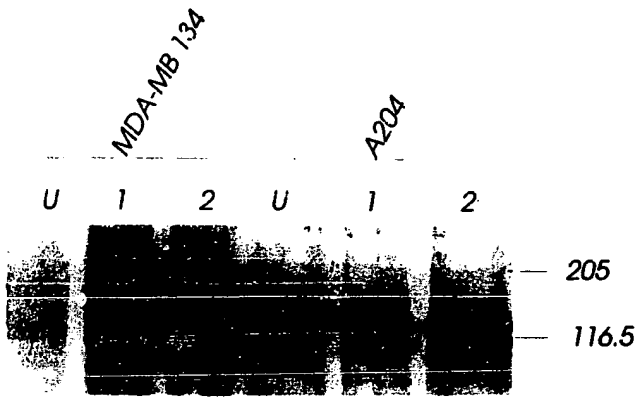

Fig. 4. FGF-1 or FGF-2 treatment of MDA-MB-134 cells results in antiphosphotyrosine immunoreactivity with a molecular size of approximately M_r 150,000. Lysates of the indicated cell lines untreated (*U*) or treated with 30 ng/ml FGF-1 plus 50 μg/ml heparin (*1*) or FGF-2 (*2*) for 30 min were subjected to SDS-polyacrylamide gel electrophoresis using a 4–20% polyacrylamide gradient gel followed by transfer to nitrocellulose membrane and probing of the blot with an antibody for phosphotyrosine. Molecular size markers are as indicated. This is a representative experiment of three.

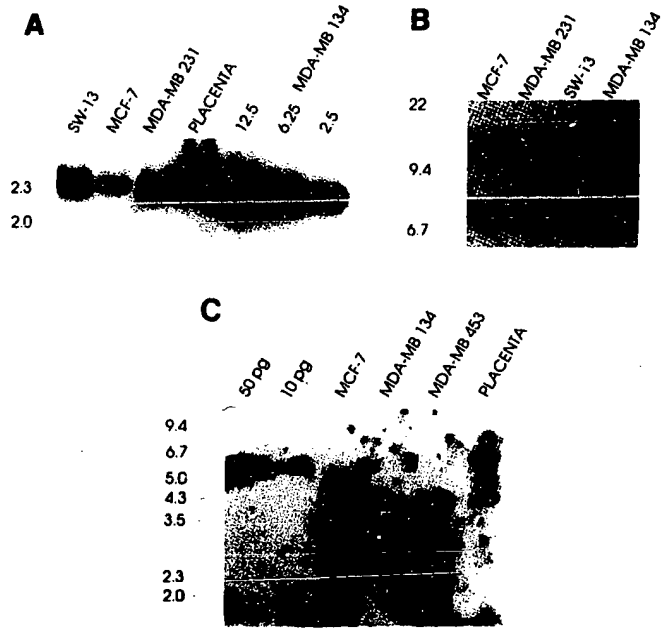

Fig. 3. The *fgfr-1* gene is amplified in MDA-MB-134 cells but the *fgfr-4* gene is not. *A* and *B*. Southern blot analysis of DNA from indicated cell lines probed with a random-primed DNA probe coding for a 355-base pair segment of the transmembrane region of *fgfr-1*. *A*, *Bam*HI digest. All *lanes* contain 25 μg DNA except for *Lanes 5–7*, which contain the indicated amounts of MDA-MB-134 DNA. *B*, *Eco*RI digest. All *lanes* contain 25 μg DNA. The approximate number of *fgfr-1* DNA copies present in MDA-MB-134 cells was estimated by comparing the intensity of the exposure of an autoradiogram similar to *A*, which also included different amounts of the linearized 3.3-kilobase pair plasmid containing the 355-base pair cDNA insert for *fgfr-1* and computing the number of copies of *fgfr-1* cDNA in a plasmid band of similar intensity to the MDA-MB 134 band. This band copy number was then divided by the number of cells necessary to obtain 25 μg DNA (the amount of DNA in the MDA-MB-134 *lane*). *C*, Southern blot analysis of a *Bam*HI digest of 25 μg DNA from indicated cell lines probed with a random-primed DNA probe for a 2.7-kilobase pair *fgfr-4* cDNA. (*Lanes 1–3* contain the indicated amounts of a 5.7-kilobase pair linearized plasmid containing the 2.7-kilobase pair cDNA sequences for *fgfr-4*.)

DISCUSSION

We have demonstrated expression of mRNA for FGFRs in all breast carcinoma cell lines tested and overexpression of FGFR mRNA in several cell lines. Twenty-two % of the cell lines we examined expressed high levels of mRNA for one or another *fgfr*. Lehtola *et al.* (32) have examined *fgfr* mRNA expression by Northern analysis in a few of these cell lines and obtained data consistent with our results. However, their study did not include overexpressing cell lines.

Since all breast carcinoma cell lines examined expressed mRNA for at least one FGFR, signaling via FGF ligand stimulation of FGF receptors is theoretically possible in all cell lines examined. We have found that MDA-MB-134 cells, which overexpress mRNA for *fgfr-1*, express high levels of mRNA for *fgfr-4*, and exhibit FGF-stimulated antiphosphotyrosine immunoreactivity, to respond to FGF treatment with growth inhibition. This is in contrast to MCF-7 breast carcinoma cells, which express low levels of mRNA for FGFRs, and have been reported to respond to FGF ligand stimulation by increased growth under some conditions (33). [The MCF-7 cells carried in our laboratory are growth-stimulated by FGF-2 and FGF-4 in estrogen-depleted medium at concentrations similar to those required for growth inhibition in MDA-MB-134 cells (data not shown)].

Overexpression of *fgfr-1* in MDA-MB-134 cells is at least partially due to gene amplification of the *fgfr-1* gene (Fig. 3). Since the gene for *fgfr-1* has been shown to be on chromosome 8, this amplification is different from the amplification of a fragment of chromosome 11 which has already been reported for these cells (28). In contrast to the FGF ligand genes contained in the chromosome 11 fragment, which do not seem to be expressed (25, 28), the gene for *fgfr-1* is transcribed with increased amounts of mRNA detectable in total RNA from these cells. In addition, MDA-MB-134 and MDA-MB-453 cells have high levels of *fgfr-4* transcripts without amplification of the gene.

We hypothesize that FGFR-1 overexpression is responsible for the growth-inhibitory response of MDA-MB-134 cells to FGFs. If this is

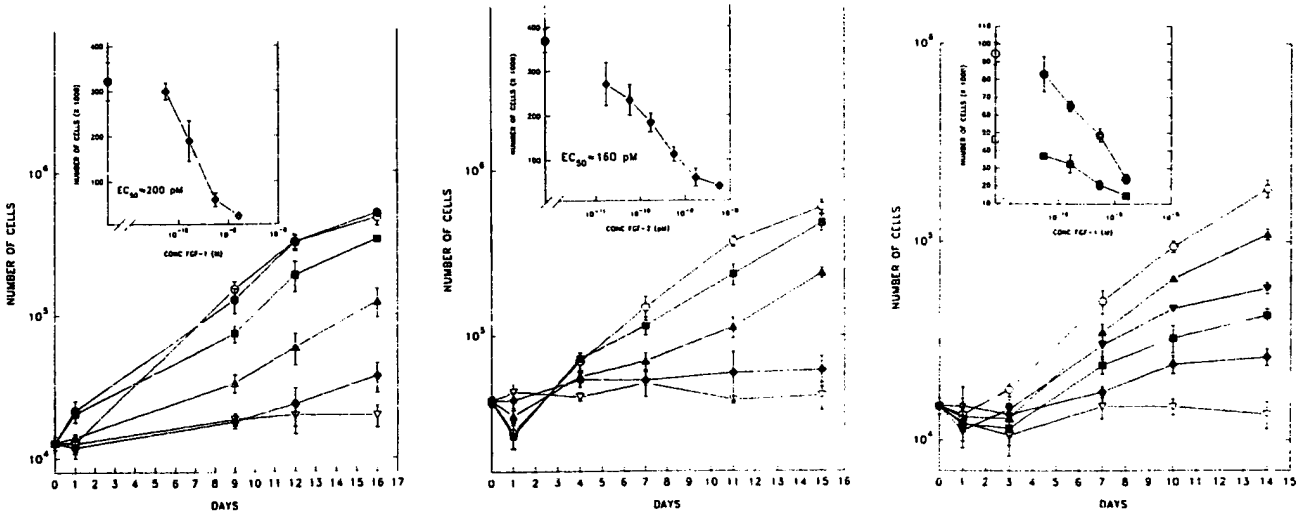

Fig. 5. FGF-1 or FGF-2 treatment of MDA-MB-134 cells results in growth inhibition. *Left*, growth of MDA-MB-134 cells with added FGF-1. (We have found heparin to be required for the growth-inhibitory effects of FGF-1 but not FGF-2. This is consistent with the data of others who find that heparin is required for the mitogenic effects (45) and binding (46) of FGF-1.) ○, IMEM with 10% FBS (16 pM FGF-1 was not different from ○ or ●); ●, plus 50 μg/ml heparin; ■, plus 54 pM (1 ng/ml) FGF-1 plus 50 μg/ml heparin; ▲, plus 162 pM (3 ng/ml) FGF-1 plus 50 μg/ml heparin; ◆, plus 540 pM (10 ng/ml) FGF-1 plus 50 μg/ml heparin; ▽, plus 1.6 nM (30 ng/ml) FGF-1 plus μg/ml heparin. *Inset*, dose-response curve for day 11. ◆, IMEM plus 10% FBS with indicated concentrations of FGF-1 plus 50 μg/ml heparin. ●, IMEM plus 10% FBS with no other additives. The approximate EC_{50} was determined by finding the half-maximal response on the ordinate and graphically determining the abscissa for this point on the dose-response curve. *Middle*, growth of MDA-MB-134 cells with added FGF-2. ○, IMEM with 10% FBS (5.7 pM and 17 pM FGF-2 were not different from ○); ■, plus 57 pM (1 ng/ml) FGF-2; ▲, plus 170 pM (3 ng/ml) FGF-2, ◆, plus 570 pM (10 ng/ml) FGF-2; ▽, plus 1.7 nM (30 ng/ml) FGF-2. *Inset*, dose-response curve for day 12. ◆, IMEM plus 10% FBS with indicated concentrations of FGF-2; ●, IMEM plus 10% FBS with no other additives. The approximate EC_{50} was determined as for *left* panel. These are representative experiments of five. *Right*, growth of MDA-MB-134 cells in 10% FCS with added FGF-1 and 50 μg/ml heparin with or without 3 nM 4-OH-tamoxifen. (Growth in IMEM plus 10% FBS with 3 nM 4-OH tamoxifen and 50 μg/ml heparin was not different from 3 nM 4-OH-tamoxifen without heparin.) ○, IMEM plus 10% FBS; ▲, plus 162 pM (3 ng/ml) FGF-1 with 50 μg/ml heparin; ▼, plus 3 nM 4-OH-tamoxifen; ■, plus 162 pM FGF-1 (3 ng/ml) plus 50 μg heparin plus 3 nM 4-OH-tamoxifen; ◆, plus 1.6 nM FGF-1 (30 ng/ml) plus 50 μg/ml heparin; ▽, plus 1.6 nM FGF-1 (30 ng/ml), plus 50 μg/ml heparin, plus 3 nM 4-OH-tamoxifen. *Inset*, dose-response curves from day 10. ●, IMEM plus 10% FBS and 50 μg/ml heparin plus indicated concentrations of FGF-1; ■, IMEM plus 10% FBS, 50 μg/ml heparin, and 3 nM 4-OH-tamoxifen plus indicated concentrations of FGF-1; ○, IMEM with 10% FBS alone; □, IMEM with 10% FBS plus 3 nM 4-OH-tamoxifen.

so, it might be expected that MDA-MB-453 cells, which overexpress mRNA for *fgfr-4*, would also be growth-inhibited by FGF-1. However, we have been unable to detect any growth response to FGF-1 in these cells (data not shown). We are also not able to detect ligand-stimulated tyrosine phosphorylation in MDA-MB-453 cells, and the number of specific high affinity FGF-1 binding sites is less than that detected in the MDA-MB-134 cell line (unpublished). It is possible that the levels of FGFR-4 are below a threshold needed for detection in the phosphotyrosine assay or that the antiphosphotyrosine antibody more readily detects activated FGFR-1 compared with FGFR-4. However, it is also possible that the *fgfr-4* mRNA overexpression observed in MDA-MB-453 cells is not carried through to protein overexpression or that there is an abnormal, nonfunctional protein. Alternatively, FGFR-4 overexpression may not lead to growth inhibition whereas FGFR-1 overexpression does. We have examined the growth of MDA-MB-453 cells in serum-free conditions and observed no effect, stimulatory or inhibitory, of FGF-1 treatment (data not shown). MDA-MB-453 cells overexpress other growth factor receptors, notably ERBB-2 (34), ERBB-3 (35), and ERBB-4 (36), which may be important in the growth of these cells. If one or more of these other receptors are determinants of growth in MDA-MB-453 cells, FGFR-4 may be a vestigial receptor which is overexpressed because it is no longer subject to regulation since it is no longer coupled to a growth-regulating pathway.

There are a number of other possible explanations for the growth-inhibitory response of MDA-MB-134 cells to FGFs besides overexpression of a functional FGFR-1 transmembrane tyrosine kinase. It is possible that expression or overexpression of a specific *fgfr-1* splice variant is responsible for the inhibitory response of MDA-MB-134 cells to FGFs. We are not able to distinguish between all of the variant *fgfr-1* transcripts or proteins at present. However, we do know that our probe will not detect mRNA for the "secreted" FGFR-1 (14) which lacks a transmembrane domain. In addition, we note two shorter protected fragments in the RNase protection assay of RNA from MDA-MB-134 and other cell lines which most likely are due to transcripts with juxtamembrane deletions as have been described (9). Thus, several FGFR-1 receptor isoforms may be present in MDA-MB-134 cells. Since all FGFR-1 membrane-bound isoforms tested thus far, as well as FGFR-4, bind FGF-1 with high affinity and FGF-1 and FGF-2 produced the same growth-inhibitory response, affinity of the specific isoforms present for particular ligands may not have a bearing on our results. It is possible that a specific receptor isoform is mediating the inhibitory growth response through its specific ability to stimulate a particular effector pathway. Better characterization of the isoforms present in MDA-MB-134 cells and transfection of antisense constructs specific for particular FGFR-1 isoforms into MDA-MB-134 cells would address this question experimentally.

By the same token, the functional responses we have observed may be mediated by FGFRs present in MDA-MB-134 cells other than FGFR-1 or FGFR-4. Transcripts from all four receptors were detected in MDA-MB-134 cells, and these cells may also express the cysteine-rich FGF receptor or other hitherto undescribed FGF receptors. However, as mentioned, MCF-7 breast carcinoma cells which also express transcripts of all four FGFRs respond to FGFs by growth stimulation, not growth inhibition.

As mentioned, a similar phenomenon is observed in MDA-MB-468 cells, which overexpress EGFR and are growth-stimulated by EGF at low doses and growth-inhibited at high doses (37). However, we do not observe such a biphasic response in MDA-MB-134 cells and were unable to reverse the FGF-stimulated growth-inhibition by addition of glucose to the medium as has been reported for MDA-MB-468 cells (18).

FGFR stimulation in some cells, such as PC-12 cells, produces a differentiated phenotype (38). We have subjected MDA-MB-134 cells to immunohistochemical analysis for markers of breast cell differentiation (E-cadherin, α-lactalbumin, and plakoglobin) as well as staining for lipid droplets and found no differences between untreated and FGF-treated cells (data not shown). In addition, cell cycle analysis by

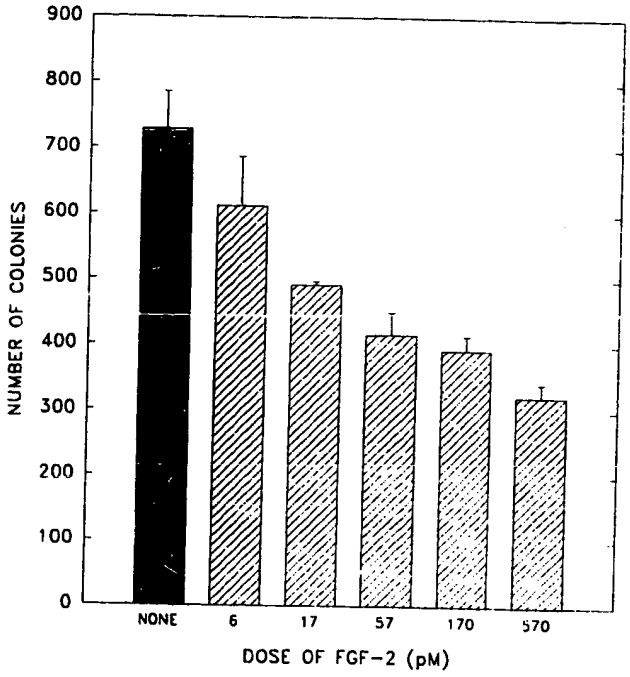

Fig. 6. FGF-2 treatment of MDA-MB-134 cells results in decreased colony formation in soft agar. Cells were plated in IMEM plus 5% FBS in 35-mm dishes in 0.36% top agar over a layer of 0.6% bottom agar at a density of 10,000 cells/dish. FGF-2 was added to the top agar in the concentrations indicated. Colonies were counted after approximately 2 weeks using an Omnicon 3600 image analysis system. The results with FGF-1 in the presence of 50 μg/ml heparin are essentially identical to those with FGF-2. This is a representative experiment of three.

a fluorescence-activated cell sorter did not reveal an increase in G_1 populations produced by FGF treatment (data not shown). Thus, the slowing of growth observed in MDA-MB-134 cells in response to FGF treatment is probably not coincident with differentiation.

Heterodimer formation has been demonstrated for FGF receptor proteins in transfection systems (11, 39, 40). Since MDA-MB-134 cells overexpress both FGFR-1 and FGFR-4 transcripts, ligand stimulation may produce FGFR-1–FGFR-4 heterodimers. Thus, a possible explanation for the inhibitory response of MDA-MB-134 cells to FGF ligand stimulation could be that FGFR-1–FGFR-4 dimers are coupled to an inhibitory pathway. This explanation would also account for the failure of MDA-MB-453 cells to be growth-inhibited by FGF-1 since MDA-MB-453 cells expressed almost no mRNA for *fgfr-1* and thus would be unable to form substantial numbers of FGFR-1–FGFR-4 heterodimers. HepG2 hepatoblastoma cells (41) as well as primary hepatocytes in culture (42) have been reported to exhibit a biphasic growth response to FGF-1 with growth-stimulation at low doses of FGF-1 and growth-inhibition at high doses. It has been suggested that, at high ligand concentrations, a low-affinity isoform of FGFR-1 is recruited which has a dominant negative effect on signal transduction produced by a higher affinity isoform (41). However, HepG2 cells express all four FGFRs (43) so that FGFR-1–FGFR-4 heterodimer formation could be occurring in these cells as well. FGFR-4 has low affinity for FGF-2 and growth-inhibition is produced by FGF-2 treatment of MDA-MB-134 cell cultures (Figs. 5 and 6). If we assume that the affinity of FGFR-4 for FGF-2 does not change upon heterodimerization with FGFR-1 and that ligand binding to both members is required for signal transduction from a heterodimer pair, one would not expect functional FGFR-1–FGFR-4 heterodimers to be produced by FGF-2 treatment of MDA-MB-134 cultures. Since the inhibitory response is present when MDA-MB-134 cell cultures are treated with FGF-2, FGFR-1–FGFR-4 heterodimers may not be mediating this response in these cells. However, since the effects of heterodimer formation on ligand affinity have not been rigorously explored, it is at least theoretically possible that heterodimer formation may alter the affinity of one member of the pair for a particular ligand.

Growth-inhibition of MDA-MB-134 cells by FGFs could be mediated through other abnormalities present in these cells which are downstream from the FGFRs. MDA-MB-134 cells have been reported to contain an activating ras^K mutation, although this appears to be present only in a subset of the population (44). In cells which are differentiated by FGFs (PC-12), RAS activation has been shown to be required downstream for the FGF effect (38). If K-RAS activation is also required as a downstream event in FGFR-mediated growth inhibition of MDA-MB-134 cells, it is unclear how FGFR stimulation could activate the oncogenic form of K-RAS further. Thus, if RAS is important as an effector of the FGF-stimulated growth-inhibitory response, it is likely that the effects we observe are on those cells having a wild-type form of the protein.

In conclusion, we have demonstrated FGFR mRNA expression in all breast carcinoma cell lines tested as well as high levels of *fgfr* mRNA expression in several cell lines. One *fgfr-1*-overexpressing cell line, MDA-MB-134, responds to FGFR stimulation by growth inhibition. At this point, we cannot differentiate between the many possible explanations for the growth-inhibitory response of MDA-MB-134 cells to FGF ligands. If FGFR-1 overexpression is responsible for the growth-inhibition, we would expect that decreasing the amount of functional FGFR-1 protein in these cells would abrogate this response and possibly lead to a growth-stimulatory response to FGF ligands. Experiments are planned, using a dominant negative receptor or antisense constructs, to test this possibility. Conversely, if a particular FGFR isoform or combination of isoforms is responsible for the growth-inhibitory response, expression or overexpression of the particular isoform(s) in a cell line that is normally stimulated by FGF ligands might change the FGF response to an inhibitory one.

While these results demonstrate the difficulty in assigning mechanistic significance to overexpression of growth factor receptors in tumors, they do point out the potential for designing therapeutic approaches to cancer involving the manipulation of growth factor responses. Further study is needed to elucidate the effector pathways responsible for the growth-inhibitory response to FGF ligands.

ACKNOWLEDGMENTS

We thank C. Acol and J. Bullocks for technical assistance. C. Sommers assisted with the differentiation studies. O. Blair and K. Creswell assisted with the FACS analysis. We thank C. Sommers, D. El-Ashry, R. Dickson, A. Carroll, L. Zhang, and M. Johnson for thoughtful reading of the manuscript.

REFERENCES

1. Harris, A. L., Nicholson, S., Sainsbury, J. R., Farndon, J., and Wright, C. Epidermal growth factor receptors in breast cancer: association with early relapse and death, poor response to hormones and interactions with *neu*. J. Steroid. Biochem., *34:* 123–131, 1989.
2. Sainsbury, J. R., Farndon, J. R., Needham, G. K., Malcom, A. J., and Harris, A. L. Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. Lancet, *1:* 1398–1402, 1987.
3. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. Human breast cancer. correlation of relapse and survival with amplification of the *HER-2/neu* oncogene. Science (Washington DC), *235:* 177–182. 1987.
4. Adnane, J., Gaudray, P., Dionne, C. A., Crumley, G., Jaye, M., Schlessinger, J., Jeanteur, P., Birnbaum, D., and Theillet, C. BEK and FLG, two receptors to members of the FGF family, are amplified in subsets of human breast cancers. Oncogene, *6:* 659–663, 1991.
5. Ding, I. Y. F., McLeskey, S. W., Chang, K., Fu, Y. M., Acol, J. C., Shou, M. T., Alitalo, K., and Kern, F. G. Expression of fibroblast growth factors (FGFs) and receptors (FGFRs) in human breast carcinomas. Proc. Am. Assoc. Cancer Res., *33:* 269, 1992.
6. Givol, D., and Yayon, A. Complexity of FGF receptors: genetic basis for structural diversity and functional specificity. FASEB J., *6:* 3362–3369, 1992.
7. Johnson, D. E., and Williams, L. T. Structural and functional diversity in the FGF receptor multigene family. Adv. Cancer Res., *60:* 1–41, 1993.
8. Partanen, J., Vainikka, S., Korhonen, J., Armstrong, E., and Alitalo, K. Diverse

receptors for fibroblast growth factors. Prog. Growth Factor. Res., *4:* 69–83, 1992.
9. Hou, J., Kan, M., McKeehan, K., McBride, G., Adams, P., and McKeehan, W. L. Fibroblast growth factor receptors from liver vary in three structural domains. Science (Washington DC), *251:* 665–668, 1991.
10. Johnson, D. E., Lu, J., Chen, H., Werner, S., and Williams, L. T. The human fibroblast growth factor receptor genes: a common structural arrangement underlies the mechanisms for generating receptor forms that differ in their third immunoglobulin domain. Mol. Cell. Biol., *11:* 4627–4634, 1991.
11. Shi, E., Kan, M., Xu, J., Wang, F., Hou, J., and McKeehan, W. L. Control of fibroblast growth factor receptor kinase signal transduction by heterodimerization of combinatorial splice variants. Mol. Cell. Biol., *13:* 3907–3918, 1993.
12. McKeehan, W. L. Complexity and heterogeneity of the FGF high affinity receptor complex. Proc. Am. Assoc. Cancer Res., *34:* 597, 1993.
13. Zimmer, Y., Givol, D., and Yayon, A. Multiple structural elements determine ligand binding of fibroblast growth factor receptors—evidence that both IG domain-2 and domain-3 define receptor specificity. J. Biol. Chem., *268:* 7899–7903, 1993.
14. Duan, D-S. R., Werner, S., and Williams, L. T. A naturally occurring secreted form of fibroblast growth factor (FGF) receptor 1 binds basic FGF in preference over acidic FGF. J. Biol. Chem., *267:* 16076–16080, 1992.
15. Miki, T., Fleming, T. P., Bottaro, D. P., Rubin, J. S., Ron, D., and Aaronson, S. A. Expression cDNA cloning of the KGF receptor by creation of a transforming autocrine loop. Science (Washington DC), *251:* 72–75, 1991.
16. Ornitz, D. M., and Leder, P. Ligand specificity and heparin dependence of fibroblast growth factor receptors 1 and 3. J. Biol. Chem., *267:* 16305–16311, 1992.
17. Burrus, L. W., Zuber, M. E., Lueddecke, B. A., and Olwin, B. B. Identification of a cysteine-rich receptor for fibroblast growth factors. Mol. Cell. Biol., *12:* 5600–5609, 1992.
18. Kaplan, O., Jaroszewski, J. W., Faustino, P. J., Zugmaier, G., Ennis, B., Lippman, M., and Cohen, J. S. Toxicity and effects of epidermal growth factor on glucose metabolism of MDA-468 human breast cancer cells. J. Biol. Chem., *265:* 13641–13649, 1990.
19. Lee, P. L., Johnson, D. E., Cousens, L. S., Fried, V. A., and Williams, L. T. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science (Washington DC), *245:* 57–60, 1989.
20. Partanen, J., Makela, T. P., Alitalo, R., Lehvaslaiho, H., and Alitalo, K. Putative tyrosine kinases expressed in K-562 human leukemia cells. Proc. Natl. Acad. Sci. USA, *87:* 8913–8917, 1990.
21. Del Sal, G., Manfioletti, G., and Schneider, C. The CTAB-DNA precipitation method: a common mini-scale preparation of template DNA from phagemids, phages, or plasmids suitable for sequencing. Biotechniques, *7:* 514–519, 1989.
22. McLeskey, S. W., Kurebayashi, J., Honig, S. F., Zwiebel, J., Lippman, M. E., Dickson, R. B., and Kern, F. G. Fibroblast growth factor 4 transfection of MCF-7 cells produces cell lines that are tumorigenic and metastatic in ovariectomized or tamoxifen-treated athymic nude mice. Cancer Res., *53:* 2168–2177, 1993.
23. Wellstein, A., Lupu, R., Zugmaier, G., Flamm, S. L., Cheville, A. L., Delli Bovi, P., Basilico, C., Lippman, M. E., and Kern, F. G. Autocrine growth stimulation by secreted K-FGF but not by endogenous basic FGF. Cell Growth & Diff., *1:* 1–5, 1990.
24. Aaronson, S. A., Bottaro, D. P., Miki, T., Ron, D., Finch, P. W., Fleming, T. P., Ahn, J., Taylor, W. G., and Rubin, J. S. Keratinocyte growth factor. A fibroblast growth factor family member with unusual target cell specificity. Ann. NY Acad. Sci., *638:* 62–77, 1991.
25. Flamm, S. L., Wellstein, A., Lupu, R., Kern, F., Lippman, M. E., and Gelmann, E. P. Expression of fibroblast growth factor peptides in normal and malignant human mammary epithelial cells. Proc. Am. Assoc. Cancer Res., *30:* 71–1989, 1989.
26. Reiner, G. C. A., and Katzenellenbogen, B. S. Characterization of estrogen and progesterone receptors and the dissociated regulation of growth and progesterone receptor stimulation by estrogen in MDA-MB-134 human breast cancer cells. Cancer Res., *46:* 1124–1131, 1986.
27. Ruta, M., Howk, R., Ricca, G., Drohan, W., Zabelshansky, M., Laureys, G., Barton, D. E., Francke, U., Schlessinger, J., and Givol, D. A novel protein tyrosine kinase gene whose expression is modulated during endothelial cell differentiation. Oncogene, *3:* 9–15, 1988.
28. Lafage, M., Nguyen, C., Szepetowski, P., Pébusque, M. J., Simonetti, J., Courtois, G., Gaudray, P., deLapeyriere, O., Jordan, B., and Birnbaum, D. The 11q13 amplicon of a mammary carcinoma cell line. Genes Chromosomes Cancer, *2:* 171–181, 1990.
29. Dionne, C. A., Crumley, G., Bellot, F., Kaplow, J. M., Searfoss, G., Ruta, M., Burgess, W. H., Jaye, M., and Schlessinger, J. Cloning and expression of two distinct high-affinity receptors cross-reacting with acidic and basic fibroblast growth factors. EMBO J., *9:* 2685–2692, 1990.
30. Ruta, M., Burgess, W., Givol, D., Epstein, J., Neiger, N., Kaplow, J., Crumley, G., Dionne, C., Jaye, M., and Schlessinger, J. Receptor for acidic fibroblast growth factor is related to the tyrosine kinase encoded by the *fms*-like gene (FLG). Proc. Natl. Acad. Sci. USA, *86:* 8722–8726, 1989.
31. Thomas, K. A. Fibroblast growth factors. FASEB J., *1:* 434–440, 1988.
32. Lehtola, L., Partanen, J., Sistonen, L., Korhonen, J., Wärri, A., Härkönen, P., Clarke, R., and Alitalo, K. Analysis of tyrosine kinase mRNAs including four FGF receptor mRNAs expressed in MCF-7 breast cancer cells. Int. J. Cancer., *50:* 598–603, 1992.
33. Briozzo, P., Badet, J., Capony, F., Pieri, I., Montcourrier, P., Barritault, D., and Rochefort, H. MCF7 mammary cancer cells respond to bFGF and internalize it following its release from extracellular matrix: a permissive role of cathepsin D. Exp. Cell Res., *194:* 252–259, 1991.
34. Yarden, Y., and Weinberg, R. A. Experimental approaches to hypothetical hormones: detection of a candidate ligand of the *neu* protooncogene. Proc. Natl. Acad. Sci. USA, *86:* 3179–3183, 1989.
35. Kraus, M. H., Fedi, P., Starks, V., Muraro, R., and Aaronson, S. A. Demonstration of ligand-dependent signaling by the *erb-B3* tyrosine kinase and its constitutive activation in human breast tumor cells. Proc. Natl. Acad. Sci. USA, *90:* 2900–2904, 1993.
36. Plowman, G. D., Culouscou, J-M., Whitney, G. S., Green, J. M., Carolton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA, *90:* 1746–1750, 1993.
37. Filmus, J., Pollak, M. N., Cailleau, R., and Buick, R. N. MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF. Biochem. Biophys. Res. Commun., *128:* 898–905, 1985.
38. Kremer, N. E., D'Arcangelo, G., Thomas, S. M., DeMarco, M., Brugge, J. S., and Halegoua, S. Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of *src* and *ras* actions. J. Cell Biol., *115:* 809–819, 1991.
39. Bellot, F., Crumley, G., Kaplow, J. M., Schlessinger, J., Jaye, M., and Dionne, C. A. Ligand-induced transphosphorylation between different FGF receptors. EMBO J., *10:* 2849–2854, 1991.
40. Ueno, H., Gunn, M., Dell, K., Tseng, A., Jr., and Williams, L. A truncated form of fibroblast growth factor receptor 1 inhibits signal transduction by multiple types of fibroblast growth factor receptor. J. Biol. Chem., *267:* 1470–1476, 1992.
41. Kan, M., DiSorbo, D., Hou, J., Hoshi, H., Mansson, P-E., and McKeehan, W. L. High and low affinity binding of heparin-binding growth factor to a 130-kDa receptor correlates with stimulation and inhibition of growth of a differentiated human hepatoma cell. J. Biol. Chem., *263:* 11306–11313, 1988.
42. Kan, M., Huang, J., Mansson, P-E., Yasumitsu, H., Carr, B., and McKeehan, W. L. Heparin-binding growth factor type 1 (acidic fibroblast growth factor): a potential biphasic autocrine and paracrine regulator of hepatocyte regeneration. Proc. Natl. Acad. Sci. USA, *86:* 7432–7436, 1989.
43. Korhonen, J., Partanen, J., Eerola, E., Vainikka, S., Ilvesmäki, V., Voutilainen, R., Julkunen, M., Mäkelä, T., and Alitalo, K. Novel human FGF receptors with distinct expression patterns. Ann. N. Y. Acad. Sci., *638:* 403–405, 1991.
44. Prosperi, M-T., Dupre, G., Lidereau, R., and Goubin, G. Point mutation at codon 12 of the Ki-*ras* gene in a primary breast carcinoma and the MDA-MB-134 human mammary carcinoma cell line. Cancer Lett., *51:* 169–174, 1990.
45. Thomas, K. A., Ortega, S., Soderman, D., Schaeffer, M-T., DiSalvo, J., Gimenez-Gallego, G., Linemeyer, D., Kelly, L., and Menke, J. Structural modifications of acidic fibroblast growth factor alter activity, stability, and heparin dependence. Ann. NY Acad. Sci., *638:* 9–17, 1991.
46. Jarvis, M. F., Gessner, G. W., Martin, G. E., Jaye, M., Ravera, M. W., and Dionne, C. A. Characterization of [^{125}I]acidic fibroblast growth factor binding to the cloned human fibroblast growth factor receptor, FGF-flg, on NIH 3T3 cell membranes: inhibitory effects of heparin, pertussis toxin and guanine nucleotides. J. Pharmacol. Exp. Ther., *263:* 253–263, 1992.